MECHANISMS OF DIAZOXIDE INDUCED PRECONDITIONING IN PRIMARY CORTICAL NEURONS.

AN ABSTRACT SUBMITTED ON THE THIRTEENTH DAY OF APRIL, 2015 TO THE NEUROSCIENCE PROGRAM IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF SCIENCE AND ENGINEERING OF TULANE UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY BY

Somhrita Dutta

Approved:

David W. Busija
(Dissertation Director)

Jill M. Daniel

Prasad V. G. Katakam

Ricardo Mostany

Jeffrey G. Tasker
Abstract

Current therapeutic options for ischemic stroke are limited to tissue plasminogen activator and mechanical clot removal therapies. Diazoxide (DZ) is a mitochondrial ATP-sensitive potassium channel opener and is protective in models of brain ischemia, but the signaling pathways involved are unknown. The mammalian target of rapamycin (mTOR) is a master regulator of protein synthesis and is involved in protection against cerebral ischemia. The neuronal nitric oxide synthase (nNOS) pathway has also been shown to provide protection from ischemic insults. Additionally, mitochondrial respiratory status has not been investigated. I examined the role of the mTOR pathway, the nNOS pathway, and mitochondrial respiration in delayed DZ-induced preconditioning of neurons.

I cultured rat primary cortical neurons and simulated ischemic stroke using oxygen-glucose deprivation (OGD) for 3 h followed by re-oxygenation. Viability, mitochondrial membrane potential, reactive oxygen species (ROS) measurements, and western blots were performed. The mTOR pathway was inhibited by rapamycin, Torin-1, and S6K targeted silencing RNA. The NOS pathway was inhibited by L-NAME. NO-donors SNP and DEANONOate (DEANO) were applied to rescue the effects of L-NAME. Mitochondrial oxygen consumption rate (OCR) was measured in intact neurons by serial injections of oligomycin, FCCP, and antimycin/rotenone.

OGD decreased viability by 50 percent, depolarized mitochondria, and reduced mitochondrial respiration whereas DZ improved viability to 75 percent and suppressed reactive oxygen species production, but did not restore
mitochondrial membrane potential after OGD. Diazoxide also increased phosphorylation of protein kinase B, mTOR, and S6K. Rapamycin, Torin-1, and S6K targeted siRNA abolished the protective effects of DZ.

Co-application of L-NAME with DZ prevented preconditioning whereas adding SNP or DEANO along with L-NAME and DZ restored protection. Diazoxide increased phosphorylated nNOS. Interestingly, co-application of L-NAME with DZ blocked the phosphorylation of nNOS as well as S6K. The ratio of phosphorylated/total Akt and mTOR were not significantly altered with L-NAME co-application.

Diazoxide altered OCR 24 and 48 h after the ischemic period. Diazoxide had no acute effect on OCR but increased ECAR significantly.

Activation of the mTOR and nNOS pathways is critical for DZ preconditioning in neurons. Furthermore, OCR is modified by the DZ-induced preconditioning of neurons.
MECHANISMS OF DIAZOXIDE INDUCED PRECONDITIONING IN PRIMARY CORTICAL NEURONS.

A DISSERTATION SUBMITTED ON THE THIRTEENTH DAY OF APRIL, 2015 TO THE NEUROSCIENCE PROGRAM IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE SCHOOL OF SCIENCE AND ENGINEERING OF TULANE UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY BY

Somhrita Dutta

Approved:

Dr. David W. Busija
(Dissertation Director)

Dr. Jill M. Daniel

Dr. Prasad V. G. Katakam

Dr. Ricardo Mostany

Dr. Jeffrey G. Tasker
Acknowledgements

When I first started this journey in 2010, I wrote an essay quoting the importance of being able to “stand on the shoulders of giants to see further”. I am very fortunate to have been able to do so in the last five years and hope that this is only the beginning.

I am forever indebted to Dr. David W. Busija, my mentor, who was patient enough to allow me to propose my own ideas, make mistakes, and guide me throughout this challenging journey. I also thank his lab members, both past and present, who have been a constant source of inspiration and encouragement for me. Dr. Ibolya Rutkai deserves special mention for her candor, endless supply of B-stress-complex vitamins, and ability to cheer me up on gloomy days. I would like to thank Nancy Busija for her discerning and meticulous edits, and for being a trusted confidante. I would like to thank the Department of Pharmacology and the Neuroscience program for their support. Additionally I would like to thank Dr. Prasad Katakam for serving on the committee and always having his door open for advice. Dr. Sarah Lindsey has always been approachable and open to me walking into her office unannounced. I would like to acknowledge Dr. Ricardo Mostany, Dr. Jill Daniel, and Dr. Jeff Tasker for their counsel and for supporting me, for always having the time to speak with me, and for being my committee members. I believe these individuals went far above and beyond their duties and their contributions towards my success are indispensable. My interactions with them beyond the formal meetings have made a difference and will always be cherished.
I have been blessed with a lot of support outside of academics from my friends and colleagues. Drs. David Brooks and Ami Raval have always been trusted colleagues. I am glad I have been able to stay in touch with friends from India: Rishika De, Arunima Chakraborty, and Trisha Banerjee. I am also fortunate to have made new friends: Daniel Simms, Noah Husek, Bryan Harter, Lauren Weber, Meghan Epstein, Mona Anchan, Loxley Battle, Cooper Battle, Molly Pucillo, Khanh Nguyen, and Samantha Hoyt. I could not ask for a better set of companions to turn to during good times and bad. As an international student, I often found myself feeling estranged no matter how long I had been in the US and in addition to my friends already mentioned, Edward Pankey and his family members have often made me feel as though I was part of something greater. I am also grateful to the education I received at Colorado College and the people I met there.

Finally, I would not have succeeded on any level without the support I have received from my family: My mother and father - thank you for the innumerable skype and phone calls and for telling me, from the other side of the world, to eat my vegetables and fruits; My brother – for always being the sane, pragmatic guide. I am thankful to have lived with a lot of animals in my life who have been a great source of comfort, entertainment, and have helped me respect animal research – my many street dogs in Calcutta, and Leo, Copper, Toby, Ninja, and Mr. Friend Cat.
## Table of Contents

### Introduction ............................................................................................................... 1

- Ischemic stroke and preconditioning .......................................................... 1
- Mitochondria and mitochondrial ATP sensitive potassium (mitoK\text{ATP})
  channels ........................................................................................................ 4
- Diazoxide as a mitoK\text{ATP} channel activator ........................................... 12
- mTOR pathway ........................................................................................... 15
- nNOS pathway ......................................................................................... 19

### Hypothesis and Aims ....................................................................................... 24

- Statement of the problem ......................................................................... 24

**Experiment 1:** The role of the mechanistic target of rapamycin (mTOR) pathway in diazoxide-induced preconditioning of primary rat cortical neurons ........................................................... 25

  - Methods .............................................................................................. 27

  - Results ................................................................................................ 34

**Experiment 2:** The role of neuronal nitric oxide synthase (nNOS) in diazoxide-induced preconditioning of primary rat cortical neurons .......... 52

  - Methods .............................................................................................. 54
Experiment 3: Characterization of mitochondrial respiration in
diazoxide-preconditioned neurons ........................................................... 63

Methods .............................................................................................. 65

Results ................................................................................................ 69

Figures

Figure 1: Simplified schematic of electron transport chain in
mitochondria .............................................................................................. 8

Figure 2: Putative structure of the mitochondrial ATP sensitive
potassium (mitoK$_{\text{ATP}}$) channel ............................................................ 10

Figure 3: The effects of diazoxide (DZ) on mitochondrial ATP
sensitive potassium (mitoK$_{\text{ATP}}$) channels ............................................. 11

Figure 4: Simplified schematic of mechanistic target of rapamycin
(mTOR) signaling pathway. ..................................................................... 17

Figure 5: Nitric oxide signaling pathway in neurons using neuronal
nitric oxide synthase (nNOS) ................................................................. 22

Figure 6: Treatment protocol for Experiment 1, adapted from Dutta et
al. 2015 (submitted) ................................................................. 26

Figure 7: Viability of neurons that were untreated or treated with
diazoxide (DZ) or a combination of DZ and rapamycin (R), wortmannin
(W), R + W, or Torin 1 (T) at normoxia .................................................. 35

Figure 8: Viability of neurons that were untreated or treated with
diazoxide (DZ) or a combination of DZ and rapamycin (R), wortmannin
(W), R+W, or Torin -1 (T) after oxygen glucose deprivation (OGD) ........ 36

Figure 9: Ratio of phosphorylated/total Akt in neurons ......................... 39

Figure 10: Ratio of phosphorylated/total mTOR in neurons ............... 40
Figure 11: Ratio of phosphorylated/total S6K in neurons ...................... 41

Figure 12: Viability of neurons at normoxia when treated with S6K-targeted siRNA ........................................................ 43

Figure 13: Viability of neurons after oxygen glucose deprivation (OGD) when treated with S6K-targeted siRNA ............................... 44

Figure 14: Effect of S6K-targeted siRNA on ratio of phosphorylated/total S6K ......................................................................... 45

Figure 15: Reactive oxygen species (ROS) production in neurons after preconditioning with diazoxide (DZ) ........................................ 47

Figure 16: Reactive Oxygen Species (ROS) production with a 15 min acute diazoxide (DZ) application ................................................. 48

Figure 17: Mitochondrial membrane potential in diazoxide (DZ) - preconditioned neurons .......................................................... 50

Figure 18: Mitochondrial membrane potential changes with an acute, 15 min diazoxide (DZ) application ......................................................... 51

Figure 19: Treatment protocol for Experiment 2 ........................................ 53

Figure 20: Viability of neurons at normoxia after modulating the neuronal nitric oxide synthase (nNOS) pathway ............................... 57

Figure 21: Viability of neurons after oxygen glucose deprivation (OGD) with similar pharmacological treatments as Figure 18 .................. 58

Figure 22: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total nNOS ................................................................. 59

Figure 23: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total Akt ................................................................. 60
Figure 24: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total mTOR ................................................................. 61

Figure 25: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total S6K ........................................................................ 62

Figure 26: Treatment protocol for Experiment 3 ........................................ 64

Figure 27: Schematic of injections and oxygen consumption rate (OCR) measurement in neurons using Seahorse Xfe24 Analyzer .......... 67

Figure 28: Representative tracing from Seahorse Mito-Stress Test on preconditioned neurons ................................................................. 70

Figure 29: Representative tracing from Seahorse Mito-stress test on preconditioned neurons 24 h after oxygen glucose deprivation (OGD) ... 71

Figure 30: Effect of oligomycin injection on oxygen consumption rate (OCR) in neurons 24 h after oxygen glucose deprivation (OGD) .......... 72

Figure 31: Effect of FCCP injection on oxygen consumption rate (OCR) in neurons 24 h after oxygen glucose deprivation (OGD) ............. 73

Figure 32: Effect of antimycin/rotenone injection on oxygen consumption rate (OCR) in neurons 24 h after oxygen glucose deprivation (OGD) ........................................................................ 74

Figure 33: Effect of oligomycin injection on oxygen consumption rate (OCR) in neurons 48 h after oxygen glucose deprivation (OGD) ........ 75

Figure 34: Effect of FCCP injection on oxygen consumption rate (OCR) in neurons 48 h after oxygen glucose deprivation (OGD) ............. 76

Figure 35: Effect of antimycin/rotenone injection on oxygen consumption rate (OCR) in neurons 48 h after oxygen glucose deprivation (OGD) ........................................................................ 77

Figure 36: Acute oxygen consumption rate (OCR) in neurons .......... 78
Figure 37: Acute extracellular acidification rate (ECAR) in neurons ... 79

Figure 38: Proposed signaling schematic of diazoxide (DZ) – preconditioning ................................................................. 92

Conclusions and Discussion ................................................................. 80

Conclusions ......................................................................................... 80

Experiment 1 ...................................................................................... 80

Experiment 2 ...................................................................................... 82

Experiment 3 ...................................................................................... 83

Limitations .......................................................................................... 87

Implications and Future Directions ......................................................... 90

References .......................................................................................... 93
Introduction

Ischemic stroke and preconditioning

A stroke is a cerebrovascular accident in which blood flow is disturbed in the brain. There are primarily two kinds of stroke: hemorrhagic and ischemic. Hemorrhagic strokes are caused by the rupture of a brain vessel while ischemic strokes, the more common type, are caused by the blockage of a cerebral vessel or hypoperfusion due to low arterial blood pressure from cardiac arrest. On the average, someone in the US has a stroke every 40 seconds and someone dies of such an event every 4 minutes (Go et al., 2014). By 2030, the American Heart Association has projected that the global prevalence of stroke will have increased by 20% in people over the age of 18. Thus, cerebral ischemia from cardiac arrest or stroke continues to be a leading cause of morbidity and mortality in the world.

Currently only one Food and Drug Administration (FDA) approved drug exists for ischemic strokes, the intra-venous delivery of tissue plasminogen activator (tPA) which can break down blood clots (Wahlgren et al., 2008; Hacke et al., 2004; Hacke et al., 2008). There are, however, several risks and limitations to this method: (1) hemorrhagic transformation of the stroke, (2) patients must be treated within 3-4.5 h from onset of symptoms, and (3) unfavorable outcomes in patients of advanced age (>80 years). Currently, only 5% of patients presenting with a stroke receive tPA treatment. The FDA also approves the use of
mechanical clot extraction alone or in combination with tPA (Berlis et al., 2004). The major limitation with this technique is that the clot must be visible and accessible so the physician can mechanically disrupt it. Therefore, there is an urgent need for novel and compatible therapies that can improve the prognosis of stroke patients.

Cerebral ischemia leads to a cascade of events that culminate in cellular death in the ischemic core as well as in the surrounding penumbra which continues after reperfusion. Immediately after stroke, the lack of oxygen and glucose leads to a deficit in ATP production. This energy failure leads to a switch to anaerobic respiration from glycolysis contributing to lactic acidosis and resulting in a decrease in the membrane potential of neurons and glia. The ionic homeostasis is destroyed, allowing ions such as calcium (Ca^{2+}) to be released into cells from organelles that usually regulate Ca^{2+}. Ion pumps are unable to remove the ions leading to Ca^{2+} overload that triggers neurotransmitter release, especially the excitatory neurotransmitter, glutamate. Simultaneously, reuptake mechanisms for neurotransmitters fail due to lack of energy. The accumulation of excess glutamate leads to more Ca^{2+} influx, as well as allowing entry of Na^+ and Cl^- ions. K+ efflux cannot counter the accumulation of Na^+ and Cl^- and fluids enter passively, leading to edema. Ca^{2+} also acts as a second messenger triggering overproduction of reactive oxygen species (ROS) and activation of enzymes such as phospholipases (which break down cell membranes), and ATPases (which breaks down ATP). The mitochondria are also damaged; the inner membrane and electron transport chain starts to fail, the mitochondrial
permeability transition pore forms leading to swelling, and further production of ROS and cytochrome c release from the mitochondria triggers apoptosis. Dying cells also release toxins into their immediate environment and pro-inflammatory mediators are produced leading to leukocyte infiltration and microglial activation (Dirnagl et al., 1999; Dirnagl, 2012).

This ischemic cascade provides opportunities for novel and experimental therapeutic intervention (Xing et al., 2012; Dirnagl et al., 1999). Upon clot removal, reperfusion injury may trigger a similar cascade of events via leukocyte infiltration, hyperperfusion, and loss of blood-brain barrier integrity (Pan et al., 2007; Dirnagl, 2012).

One experimental therapy for stroke involves ischemic preconditioning (IPC), in which a brief sub-lethal ischemic insult protects against a subsequent lethal ischemic attack. In the late 1980s, researchers found that occluding a major artery for a few minutes before exposing an animal to an experimental heart-attack reduced damage from heart-attack (Murry et al., 1986). This same effect was observed in the brain (Kitagawa et al., 1991; Kitagawa et al., 1990).

Classically, IPC offers two windows of protection: early and delayed. The early window of protection begins minutes after IPC and lasts for a few hours, affecting ion channels and post-translational modifications of proteins. The delayed, and often the more robust phase can be protective for 2 to 3 days following IPC and is mediated by synthesis of new proteins and altered gene expression. Understanding the signaling mechanisms which are triggered after IPC has been an important research endeavor. For example, IPC has been shown to
upregulate nuclear factor erythroid-2 related factor and STAT3, both of which can attenuate ischemic stress (Thompson et al., 2014). Additionally, cellular signaling pathways such as hypoxia-inducible factor-1, SIRT1, and PKCε have been explored (Thompson et al., 2012). Recent research from Thompson and colleagues has shown that IPC modifies epigenetics by reducing DNA methylation, inhibiting histone deacetylases, and elevated SUMOylation (Thompson et al., 2013).

Similarly, several studies have focused on finding pharmacological agents that can be used as a preconditioning treatment. Anesthetics, sirtuin 1 activators, and mitochondrial ATP-sensitive potassium (mitoKATP) channel activators have all been shown to be effective preconditioning agents against cerebral ischemia. Specifically, Domoki and colleagues showed that diazoxide (DZ) can be used to precondition the brain against stroke (Domoki et al., 1999). However, the mechanisms underlying DZ’s protective effect continue to be undefined.

*Mitochondria and Mitochondrial ATP-sensitive potassium (mitoKATP) channels*

Mitochondria are double-membraned, dynamic organelles that are also responsible for energy production via ATP synthesis. The basic structure of a mitochondrion is comprised of an outer and inner membrane and in-foldings, called cristae, which increase the surface area for oxidative enzyme reactions (Guyton and Hall, 2011). There is a plethora of evidence that mitochondria can fuse and divide to meet the changing energy needs of
a cell (Wappler et al., 2013). Mitochondria are also the only cellular organelle, other than the nucleus, that has its own DNA, leading to its self-replicative nature. It also maintains the most negative membrane potential of all organelles (-120 to -180mV). In addition to producing energy, mitochondria sequester calcium, produce ROS, and are involved in programmed cell-death. All of these factors make mitochondria an important organelle to study. An increasing number of diseases have been shown to have a mitochondrial basis, including schizophrenia and diabetes.

The mitochondria are prominent structures within a cell that can produce high energy ATP using oxidative phosphorylation. Fats, sugars, and proteins in the cytoplasm are broken down to pyruvate via glycolysis. Pyruvate is converted to acetyl-coenzyme A (acetyl-coA) which enters the citric acid/tricarboxylic/Krebs/TCA cycle in the mitochondrial matrix. Acetyl-coA undergoes a series of reactions decomposing into carbon dioxide and hydrogen ions. The TCA cycle also generates nicotinamide adenine dinucleotide (NADH) and succinate.

Complexes I, II, III, IV, and V (Figure 1) are contained in the inner membrane of the mitochondria. Complexes I, III, and V are proton pumps. Together with the TCA cycle, they constitute the electron transport chain. NADH and succinate donate electrons to complexes I and II, respectively. Electrons are sequentially transferred to coenzyme Q (a lipid-soluble electron carrier), complex III, cytochrome c (a water-soluble electron carrier), and complex IV, and finally to oxygen which is reduced to water. The proton
gradient formed on the inner mitochondrial membrane is used by ATP synthase (complex V) to generate ATP from ADP and inorganic phosphate via oxidative phosphorylation. ADP and ATP are unable to diffuse freely in or out of the mitochondria, but a specialized transporter protein called ATP-ADP translocase enables these molecules to pass through the mitochondrial membrane. The transport of ATP into the cytosol is always coupled to the entry of ADP into the mitochondria (Berg et al., 2002). The electron transport chain results in production of superoxide (O$_2^-$), one of the major mitochondrial ROS, and is constantly produced and rapidly converted to either H$_2$O$_2$, by MnSOD, or to peroxynitrite (a reactive nitrogen species or RNS) by reacting with NO. Catalase and glutathione (GSH) may decompose the H$_2$O$_2$ to water and oxygen. In general, basal levels of ROS and RNS are essential for normal signaling within the mitochondria, but excess ROS and RNS can lead to deleterious effects. At physiological levels, ROS and RNS regulate inflammation, cell cycle, and survival signaling pathways. Additionally, ROS and RNS production is tightly regulated by antioxidants present within the system to avoid oxidative stress. The primary sites of superoxide production in the electron transport chain are complexes I, II, and III. ROS may be generated due to reduced electron acceptance, thus allowing electrons to react with molecular oxygen, or due to reverse electron transport between complexes (Murphy, 2009; Turrens, 2003; Drose et al., 2011). When the antioxidant system is overwhelmed by overproduction of ROS or RNS, harmful signaling pathways are activated. For example, with its
limited repair capabilities, mitochondrial DNA is particularly vulnerable to oxidative stress due to excess ROS production (Kujoth et al., 2005). Oxidative stress can also lead to release of cytochrome c and activation of the apoptotic pathway via opening of the mitochondrial permeability transition pore.
Figure 1: Simplified schematic of electron transport chain in mitochondria. Reduced nicotinamide adenine dinucleotide (NADH) produced from the Tricarboxylic Acid cycle donates electrons to complex I. Succinate donates electrons to complex II. NADH is oxidized to NAD⁺ while succinate is oxidized to fumarate. Ubiquinone (Q) transfers electrons to complex III. Complex III transfers electrons to complex IV via cytochrome C. Complex IV uses the electrons and hydrogen ions to reduce molecular oxygen to water. The proton gradient is used to phosphorylate ADP to ATP.
The existence of the mitoKATP channels is a controversial subject (Dos Santos et al., 2002; Costa et al., 2006). Located on the inner membrane of mitochondria (Inoue et al., 1991), activation of these channels leads to depolarization of the mitochondria allowing influx of K⁺ ions. In contrast, plasmalemmal KATP channels lead to hyperpolarization of the cell. The mitoKATP channels have become important potential protective targets against ischemic and chemical stress. Lacza and colleagues explored the major composition of the mitoKATP channels and found evidence of pore-forming Kir 6.1 and Kir 6.2 subunits as well as a SUR-2 like receptor (Figure 2) (Lacza et al., 2003a). Interestingly, Fedele and colleagues recently observed significant specific polymorphisms of Kir 6.2 in patients undergoing coronary angiography, indicating a correlation between this subunit and a clinical susceptibility for cardiac dysfunction (Fedele et al., 2013). The mitoKATP channels are activated by physiological stimuli like GTP, GDP, superoxide anion, and peroxynitrite and are inhibited by ATP and ADP. Pharmacologically, both DZ and BMS-191095 effectively activate mitoKATP channels while cromakalim, nicorandil, and pinacidil are known to activate plasmalemmal and mitoKATP. Compounds including 5-Hydroxydecanoate (5-HD) and glibenclamide are pharmacological blockers of mitoKATP and KATP channels, respectively.
Figure 2: Putative structure of the mitochondrial ATP sensitive potassium (mitoK\textsubscript{ATP}) channel. GTP, GDP, superoxide (O\textsubscript{2}\textsuperscript{-}), and peroxynitrite (ONOO\textsuperscript{-}) are endogenous activators while ATP and ADP are endogenous inhibitors of mitoK\textsubscript{ATP} channels. Pharmacological activators include cromakalim, BMS 191095, and the putative opener, DZ, while 5-HD is the pharmacological inhibitor.
**Figure 3:** The effects of diazoxide (DZ) on mitochondrial ATP sensitive potassium (mitoK$_{ATP}$) channels. Diazoxide opens the mitoK$_{ATP}$ channels and allows influx of potassium ions, depolarizing the mitochondria. In addition, DZ has a known effect of inhibiting complex II. This can lead to ROS formation.
**Diazoxide as a mitoK\textsubscript{ATP} channel activator**

Diazoxide has been shown to be protective in several *in vitro* and *in vivo* models of ischemic/anoxic injury via activation of the mitoK\textsubscript{ATP} channels (Figure 3). Pioneering studies from Dr. Busija’s laboratory showed that topical application of 5-10µM DZ on the surface of the piglet cortex protected the brain from 10 min of global ischemia, and the effect was blocked by 5-HD (Domoki et al., 1999). Diazoxide pre-treatment also reduced infarct volume in adult rodents undergoing middle cerebral artery occlusion (MCAO) (Shimizu et al., 2002) and prevented neuronal cell death in a neonatal model of hypoxia-ischemia (Rajapakse et al., 2002); 5-HD abolished the effect in both cases. Rats pre-treated with DZ also showed reduced edema and blood-brain-barrier permeability (Lenzser et al., 2005). Intraperitoneal administration of DZ 24 h before MCAO showed reduced infarct size 72 h after reperfusion. The protective preconditioning was prevented by 5-HD (Mayanagi et al., 2007a). *In vitro* studies showed that DZ pre-treatment preconditions astrocytes against insults such as oxygen-glucose deprivation (OGD) and hydrogen peroxide toxicity (Rajapakse et al., 2003). Other findings from this study showed that protection was abolished by 5-HD and ROS production stimulated by DZ was reduced by use of antioxidants and increased phosphorylation of protein kinase C (PKC). A subsequent study showed that DZ preconditioned neurons in culture and protected against OGD (Kis et al., 2003b), and the protection was abolished by 5-HD, PKC inhibitor chelerythrine, and superoxide dismutase (SOD) mimetic M040401.
When exposed to toxic levels of glutamate, DZ-preconditioned neurons produced less ROS compared with untreated neurons and the preconditioning effect was blocked by M040401 (Nagy et al., 2004).

To understand the mechanism behind protection, Domoki and colleagues (2004) explored mitochondrial calcium accumulation and mitochondrial swelling in piglet brains following hypoxia-ischemia. They found that both were attenuated by DZ-pretreatment and, as before, the effect was abolished by 5-HD. In a piglet model of hypercapnia after ischemia, DZ preserved vasodilatory status of pial arteries (Domoki et al., 2005). A later study by Katakam et al. (2007) using the Zucker Obese (ZO) model of insulin resistance, showed that DZ failed to precondition the heart against ischemia reperfusion injury, suggesting that some disease states change mitochondria significantly, causing ineffective preconditioning by DZ. Further investigation (Katakam et al., 2009) revealed that cerebral arteries of these ZO rats exhibited reduced dilation to DZ, suggesting a fundamental mitochondrial impairment. Katakam et al. showed that BMS-191095 mediated vasodilation, calcium sparks, and mitochondrial depolarization were all diminished in ZO rats. These studies suggest DZ and BMS-191095 responses are impaired in certain pathological conditions. Recently, our lab compared the mitochondria-mediated responses in cerebral vasculature after MCAO in contralateral vs. ipsilateral hemispheres (Rutkai et al., 2014). Diazoxide-mediated dilatory responses were maintained 4 h post-MCAO. At 48 h post-MCAO, DZ-mediated responses on the contralateral side were
similar to control, but the ipsilateral response was diminished. This important finding suggests that the two hemispheres respond differently after stroke, that post-ischemic injury is a dynamic process, and that mitochondria can be a viable target after stroke.

Another mitoK_{ATP} activator, BMS-191095, has been shown to have similar effects. Busija and colleagues investigated the effects of DZ and BMS191095 on isolated piglet mitochondria (2005). Both compounds decreased mitochondrial membrane potential, but BMS191095 was a more selective agent and did not produce the ROS seen with DZ. Previously, DZ was shown to inhibit succinate dehydrogenase (Kis et al., 2003b) and succinate dehydrogenase inhibitor 3-nitropropanoic acid increased ROS production but did not change $\Delta \Psi_m$. Both glibenclamide and 5-HD blocked DZ and BMS191095-mediated effects of $\Delta \Psi_m$ and DZ’s enhancement of ROS production. In rats, pre-treatment with BMS191095 24 h before MCAO also led to a delayed preconditioning as shown by reduced infarct volume. In the same study, cultured neurons also showed mitochondrial depolarization with ROS production. All observations were abolished when 5-HD was used (Mayanagi et al., 2007b). Another seminal study directly compared the two mitoK_{ATP} openers (Katakam et al., 2013). Both DZ and BMS191095 increased intracellular calcium levels and stimulated NO release via the PI3K-Akt-eNOS pathway. However, only DZ had a noticeable effect on ROS production, suggesting two distinct pathways of mitochondrial activation.

These studies indicate that our understanding of the mechanism behind DZ’s
acute effects and pre- and post-conditioning effects is constantly evolving. It also shows that the drug has a myriad of complex effects that could shed more light on mitochondria as a target in ischemic strokes.

The mTOR pathway

The mTOR pathway has recently emerged as a master regulator of cell metabolism, autophagy, and mitochondrial metabolism (Laplante and Sabatini, 2012). The main role of the mTOR kinases is to integrate extracellular information with metabolic, anabolic, and catabolic processes. As shown in figure 4, two forms of mTOR exist: complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 is very sensitive to the energy status of the cell and is considered to be rapamycin (R) sensitive. However, with longer R treatment, mTORC2 is also affected. mTORC1 phosphorylates elf-4E binding protein and p70 ribosomal S6 kinase (S6K), which are largely responsible for the molecular mechanism of mTOR’s actions. mTORC2 modulates Akt and PKCα activity. Much excitement has been generated since ketamine’s acute antidepressant effects via synaptogenesis were shown to be mediated by the mTOR pathway (Li et al., 2010b). Many investigators have shown that mTOR activation is important to cell survival during ischemia (Mao et al., 2013; Wang et al., 2012; Xie et al., 2013; Chen et al., 2012b). However, the literature is in debate about whether mTOR activation is detrimental or beneficial (Maiese, 2014; Pignataro et al., 2011). In the adult brain, mTOR is reported to be crucial for learning and long-term memory and activation of mTOR has been shown to not only protect neurons against global brain ischemia but also to preserve memory function, which may
have significant implications regarding quality of life after a stroke (Zare Mehrjerdi et al., 2013).
Figure 4: Simplified schematic of mechanistic target of rapamycin (mTOR) signaling pathway. We investigated the part of the pathway highlighted in red text. mTOR is assembled into two complexes: complex 1 (mTORC1) and 2 (mTORC2). Rapamycin is believed to inhibit mTORC1; however, long-term treatment with rapamycin inhibits mTOR assembly and thus affects both complexes. PI3K/Akt activated mTORC1 which in turn phosphorylates S6Kinase (S6K), eukaryotic translation initiation factor 4E binding protein (4E-BP1), and autophagy-related protein 13 (ATG-13). The mTORC2 can also activate Akt by phosphorylation.
Evidence also suggests that multiple neuroprotective pathways work via phosphorylation of Akt and S6K, the key upstream and downstream targets of mTOR, respectively. In addition, melatonin has been shown to be neuroprotective in stroke models in vitro in a hippocampal cell line as well as in vivo using MCAO via the activation of the mTOR pathway (Koh, 2008). Deleting the phosphatase and tensin homolog on chromosome 10 attenuated neuronal apoptosis via phosphorylation of mTOR in an in vivo model of ischemia (Shi et al., 2011). The mTOR pathway regulated key factors in neuronal survival: the hypoxia-inducible factor alpha and vascular endothelial growth factor (Chen et al., 2012b). Kis, Yellon, and Baxter (2003a) showed that IPC in cardiac tissue induces delayed cardioprotection in rabbit hearts via activation of the mTOR pathway. Pastor and colleagues (2009) also showed that mTOR-S6K pathway is crucial to protection against stroke in astrocytes derived from S6K knock-out mice and in vivo in the S6K knock-out mice. Even so, the relationships between DZ preconditioning and the mTOR pathway have remained unexplored in neurons.

Rapamycin is the classical pharmacological inhibitor of mTOR which was originally developed as an antifungal agent and is now used for its anti-proliferative and immunosuppressive effects. The major effect of R is on mTORC1 by inhibiting FK – Binding Protein 12 and binding to mTORC1. Rapamycin also allows mTORC2 to phosphorylate Akt leading to residual activation of the Akt pathway.
Torin-1 (T) is a newer compound that is an ATP-competitive catalytic inhibitor of mTOR which inhibits both mTORC1 and mTORC2 and thus there is no feedback phosphorylation of Akt using T, making T a more complete and specific pharmacological inhibitor of the mTOR complexes.

I used wortmannin (W) to further confirm the linearity of the pathway. Wortmannin covalently binds and inhibits PI3K and has been shown to also inhibit downstream complexes such as mTOR.

*The nNOS pathway*

Nitric oxide (NO) can freely diffuse into neighboring cells and organelles, including the mitochondria, and consequently can alter mitochondrial-based signaling pathways. Since NO performs important physiological functions (neurotransmission, maintenance of vascular tone), reduced bioavailability can be detrimental to normal physiological processes (Forstermann and Sessa, 2012). This reduced bioavailability can arise from a decreased expression of nitric oxide synthase (NOS) or by the uncoupling of NOS, leading to superoxide formation and the subsequent reaction of superoxide with NO to form peroxynitrite (ONOO⁻) (Sullivan and Pollock, 2006). Research into the NOS uncoupling mechanism has revealed that electrons may flow to molecular oxygen instead of flowing to L-arginine to catalyze NO formation. A deficiency in tetrahydrobiopterin, or L-arginine, as well as dephosphorylation of NOS can all lead to uncoupling.

Our lab has demonstrated that NO/ONOO⁻ acts as an endogenous activator of the mitoK_{ATP} channel (Lacza et al., 2003b) and that depolarizing
the mitochondria in cerebral endothelial cells using DZ can activate endothelial NOS (eNOS) and allow vasodilation (Katakam et al., 2013). Additionally, over-activation and uncoupling of nNOS is thought to be the main culprit of NMDA receptor-mediated cell death and excitotoxicity in stroke (Lipton et al., 1993) due to enhanced ROS production, which also occurs in the cultured neurons that I am using. nNOS knockout mice are resistant to ischemic insult (Huang et al., 1994) and inducible NOS contributes to tissue damage and cell death (Iadecola et al., 1995). However, eNOS mediates vasodilation and exerts a protective effect during ischemia and eNOS knockout mice have larger infarct sizes compared with wild-type mice (Huang et al., 1996). Thus, there are deleterious as well as protective effects of NO depending on the source (Huang, 2004).

A well-studied signaling cascade which activates nNOS in neurons is via NMDA receptors (Figure 5). Glutamate is released from the presynaptic terminal and acts upon NMDA receptors in the postsynaptic element. Opening of the channel allows calcium to enter and activate NOS through calmodulin, thus forming a calcium-calmodulin complex. This complex, along with tetrahydrobiopterin, binds to nNOS and translocates it to the cytosol. Once in the cytosol, nNOS is dephosphorylated by calcineurin, initiating the production of NO. L-arginine is converted to L-citrulline in the presence of nNOS producing NO. Consequently, NO activates guanylyl cyclase and activates the various cGMP-regulated signaling pathways. Neuronal NOS is
inactivated by phosphorylation by protein kinase A or protein kinase C (PKC).
**Figure 5:** Nitric oxide signaling pathway in neurons using neuronal nitric oxide synthase (nNOS). A well-studied way of activating nNOS is via the activation of NMDA receptors. Glutamate from pre-synaptic terminals is released allowing calcium to go into the cell. Calcium and calmodulin form a complex which along with calcineurin and tetrahydrobiopterin activates nNOS and translocates it to the cytosol. In the cytosol, L-arginine is converted to L-citrulline which produces nitric oxide.
Our lab has previously shown that, due to repetitive cortical spreading depression, brain preconditioning *in vivo* requires a critical step involving NO production (Horiguchi et al., 2005). However, in the intact brain, whether NO is involved in preconditioning due to DZ administration has never been examined. Thus, I have explored the role that nNOS/NO neuron signaling plays in DZ preconditioning and ischemia.

In our studies, I used $N_\omega$-Nitro-L-arginine methyl ester hydrochloride (L-NAME) which must be hydrolyzed to $N_\omega$-Nitro-L-arginine (L-NNA) to become an active inhibitor of NOS. L-NNA competes with L-arginine to bind with NOS and produce NO. I also used sodium nitroprusside (SNP) to exogenously supply NO because SNP is destabilized in solution and releases NO. Diethylamine NONOate (DEANO) was also used as an NO donor because it spontaneously dissociates to produce NO in a pH-dependent manner.
Hypothesis and Aims

Statement of the problem

My goal was to elucidate the signaling pathways triggered by DZ induced preconditioning in rat embryo-derived primary cortical neurons to find better pharmacological targets for new stroke treatments. I investigated some key regulators of cell-signaling: the mTOR pathway, the nNOS pathway, and assessed mitochondrial respiration in the neurons.

In all experiments I modeled an in vitro stroke using OGD where I removed the regular media for neurons and replaced it with glucose-free DMEM. To mimic an oxygen free environment, neurons were kept in an anaerobic chamber. This method is described in further detail later.
**Experiment 1: To investigate the role of the mechanistic target of R (mTOR) pathway in DZ-induced preconditioning of primary rat cortical neurons.**

In Experiment 1, I tested the hypothesis that DZ induced preconditioning in neurons is mediated by the mTOR pathway by pharmacologically inhibiting mTOR using R and T. I inhibited the major upstream and downstream kinase of mTOR using W (for Akt) and S6-Kinase-specific siRNA for S6K. I assessed cellular viability and protein expression of the pathway of interest (phosphorylated Akt/total Akt, phosphorylated mTOR/total mTOR, and phosphorylated S6K/total S6K). I also evaluated reactive oxygen species (ROS) production and mitochondrial membrane potential with DZ-treatment to see how modulating the mTOR pathway affects these parameters (Figure 6).
Figure 6: Treatment protocol for Experiment 1, adapted from Dutta et al. 2015 (submitted). Primary neurons were cultured from E-18 rats. After 6 days, neurons were treated for 3 days consecutively with diazoxide, rapamycin, wortmannin, diazoxide + wortmannin, diazoxide + rapamycin, diazoxide + Torin-1, or diazoxide + S6K siRNA. On DIV 7 the siRNA treatment was applied for 4 h before diazoxide was added. After this, cells were placed in an anaerobic chamber to mimic an ischemic stroke. During reoxygenation, cells were collected for ROS and mitochondrial membrane potential measurements, viability assays, and western blots.
Methods

Sterile Technique:

All instruments used for cell culture were autoclaved before and after each use to maintain sterile technique. All instruments, pipette tips, media, dishes, plates, and other supplies used for cell culture were opened under a hood. The hood was sterilized after each use with 70% ethanol and once a week with ultraviolet light for 1 h.

Animals:

Pregnant, E-18, Sprague Dawley rats and their embryos (average of 10 per dam) were obtained from Harlan (Indianapolis, IN). Their use for these studies was approved by the Tulane University School of Medicine Animal Care and Ethics Committee. Animals were given access to food and water ad libitum.

Rat Primary Cortical Neuronal Cell Culture:

Rats were deeply anesthetized with isoflurane in a bell-jar and decapitated and the uterine cavity was exposed. Primary rat cortical neurons were isolated from the heads of E18 rat fetuses, as described previously (Deadwyler et al., 1993; Kis et al., 2003b). The cranium of each embryo was collected in calcium and magnesium free PBS. For each cranium, autoclave–sterilized scissors and forceps were used to extract the cerebrum from the skull, and meninges and blood vessels were removed by rolling on a filter paper. The brains were then placed in a 6 mm dish containing 5 ml of “isolation buffer” (containing DMEM, 1% Penicillin-Streptomycin and 0.1% Gentamycin solution) and washed twice with
isolation buffer using a Pasteur pipette. After the last wash, all isolation buffer was removed and the digestion enzyme was prepared (1 ml Dispase and 20 ul DNAse). Cortices were added to the digestion enzyme and the Eppendorf tube was placed in a water-bath warmed to 37° C for 34 min. Digestion was stopped by centrifuging the tube, removing supernatant, and adding 1 ml isolation buffer. After digestion and trituration, isolated cells were plated onto poly-D-lysine coated plates or dishes in a plating medium consisting of 60% DMEM, 20% F-12 HAM, 20% horse serum, and L-glutamine (0.5 mM). Cultures were maintained in a humidified 5% CO2 incubator. After cell attachment, the plating medium was replaced with Neurobasal medium supplemented with B27 (2%), L-glutamine (0.5 mM), 2-mercaptoethanol (55 μM), and KCl (25 mM). Positive immunostaining for microtubule-associated protein-2 and negative immunostaining for glial fibrillary acidic protein showed that the cultures consisted of more than 99% neurons.

Materials:

Diazoxide (500 μM, Sigma Aldrich, St Louis, MO, USA) was used to induce preconditioning, as described previously (Kis et al., 2003b). Rapamycin (400 nM, Sigma Aldrich) and T (2 nM, Tocris Biosciences, Bristol, United Kingdom) was used to inhibit the mTOR pathway. Diazoxide was dissolved in 0.1M NaOH and the other drugs were dissolved in dimethyl sulfoxide (DMSO). Vehicle controls were performed for each type of experiment. S6K targeted siRNA was purchased from Invitrogen (Grand Island, NY, USA) and dissolved in RNAse free water.
**Experimental Design:**

For Experiment 1, primary cortical neurons were cultured for 7 days *in vitro* (DIV). On the 7th day, cells were treated with (1) Feeding Medium (FM), (2) DZ, (3) R, (4) DZ+R, (5) DZ+T, or (6) DZ+S6K-targeted siRNA (DZ+S6K siRNA) for 3 consecutive days. On DIV 10, neurons were either subjected to OGD or maintained at normoxia, as described below. Mitochondrial membrane potential and ROS measurements were conducted immediately after OGD. Lysates were collected after 2 h of reoxygenation for Western blotting and viability assays and mitochondrial respiration assays were performed 24 h after reoxygenation.

**Oxygen Glucose Deprivation:**

Briefly, 96-well cell culture plates, 3mm dishes, and poly-D-lysine coated Seahorse Xfe 24-well plates were rinsed twice with PBS and the culture medium was replaced with glucose-free DMEM (Wappler et al., 2013). Cultured neurons were placed in a Shel Lab Bactron Anaerobic Chamber (Sheldon Manufacturing, Cornelius, OR, USA) containing anaerobic mixed gas (AMG; 5% CO\(_2\) - 5% H\(_2\) - 90% N\(_2\)) at 37°C for 3 h. The 5% H\(_2\) in the AMG removed the remaining traces of oxygen-forming water on a platinum catalyst. The oxygen level was continuously monitored with an infrared gas analyzer (Illinois Instruments, Ingleside, IL, USA) and maintained at <0.1% during the experiments. Control cell cultures were treated identically and incubated in glucose-containing DMEM (4.5 mg/ml) in a 5% CO\(_2\) cell culture incubator. OGD was terminated by removing the cell culture plates and dishes from the anoxic chamber and replacing the glucose-free
DMEM with regular culture medium containing glucose. The cells were then maintained in a 5% CO₂ incubator until determination of cell viability.

**Quantification of cellular viability assay:**

Twenty-four h after OGD, cell viability was assessed using the tetrazolium-based CellTiter 96 AQueous One Solution Assay (Promega, Madison, WI, USA). Twenty microliters of solution was carefully pipetted into the culture wells. Cultures were incubated for 1 h at 37 °C, during which media turned from yellow to brown, followed by measurement of absorbance at $\lambda_{\text{abs}} = 492$ nm with a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH, Offenburg, Germany). Results were compared with paired cultures exposed to the same neurotoxic stimulus on the same day and cell viability was expressed as the percentage of the corresponding control culture (untreated and not exposed to the lethal insult) using the following formula:

$$\% \text{viability}_{\text{SAMPLE}} = \frac{(\text{absorbance}_{\text{SAMPLE}} - \text{absorbance}_{\text{BACKGROUND}}) \times 100}{(\text{absorbance}_{\text{CONTROL}} - \text{absorbance}_{\text{BACKGROUND}})}.$$ 

**Western blots:**  

Proteins were harvested by scraping neurons from the dishes in ice-cold NP40 lysis buffer (Invitrogen, Grand Island, NY, USA) supplemented with proteinase and phosphatase inhibitors and then homogenized and stored for Bradford protein estimation (Sigma Aldrich) (each 5 µl/mL). Cell lysates were resolved using standard denaturing conditions: cells were homogenized and equal amounts of protein (30 µg) from the whole cell lysates were incubated with sodium dodecyl sulfate (SDS)/β-mercaptoethanol sample buffer at 100°C for 5
min. Protein samples were separated by electrophoresis on a 4–20% SDS-PAGE gradient gel at 100V for 2 h, and proteins were transferred onto a nitrocellulose or PVDF membrane (75 V for 2 h) and incubated in a Tris-buffered saline containing 0.1% Tween 20 (TBST) with 5% Bovine Serum Albumin or milk blocking solution for 1 h at room temperature followed by incubation with primary antibodies overnight at 4°C in the blocking solution. The membranes were washed three times in TBST and incubated for 1 h in the blocking buffer with goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-mouse IgG (1:5000, Santa Cruz) conjugated to horseradish peroxidase.

Protein levels were determined using the following primary antibodies: phosphorylated Akt \(^{\text{Ser}473}\) (p-Akt \(^{\text{Ser}473}\), 60 KDa, Cell Signaling, Danvers, MA), total Akt (60 KDa, Cell Signaling), phosphorylated mTOR \(^{\text{Ser}2448}\) (p-mTOR \(^{\text{Ser}2448}\), 298 KDa, Cell Signaling), total mTOR (289 KDa, Cell Signaling), phosphorylated S6K \(^{\text{Thr}389}\) (p-S6K \(^{\text{Thr}389}\), 70 and 85 KDa, Cell Signaling), and total S6K (70 and 85 KDa, Cell Signaling). Chemiluminescence and autoradiography was used to visualize the final reaction of the primary antibodies and their respective horseradish peroxidase conjugated secondary antibody. \(\beta\)-actin was used as a house-keeping protein to ensure equal loading. Western blots for Akt and mTOR showed a single band at the appropriate molecular weight whereas two bands at appropriate molecular weights were present for S6K. I included both bands for S6K in our analyses.

For quantitative analysis, the immunobands were scanned and band intensities were quantified using Image J 1.3.1 software. Band intensities were
normalized to β-actin (1:5000, Sigma Aldrich) and the control group’s normalized protein levels were considered as 100%.

**siRNA transfection:**

Lipofectamine RNAiMax was used with silencer select siRNA targeting p70S6K (cat #4390771, ID: 29105) to transfect neurons on DIV 7. Cells were washed twice with Opti MEM media. Lipofectamine RNAiMax and siRNA were dissolved in Opti MEM. Recommended dilutions of the siRNA (25 – 50 pmol) and lipofectamine mixtures were incubated at room temperature for 5 min and then added to cultured neurons plated in dishes or 96-well plates for 4 h before resuming pharmacological treatment with DZ. Lipofectamine RNAiMax with Opti MEM was used as a vehicle control. The negative control (cat #4390843) was non-targeting siRNA with comparable chemical modifications as the S6K targeted siRNA. To confirm knock downs, I performed Western blots after 72 h of transfection. I also conducted viability assays 24 h after OGD.

**Mitochondrial membrane potential (ΔΨᵢ) (Rhodamine Assay):**

The ΔΨᵢ was analyzed using the Rhodamine 123, as previously described (Carvalho et al., 2014). Neuronal cultures in 96-well plates were loaded in the dark with Rhodamine (0.025 nM) in a 5% CO₂ incubator (for cells maintained at normoxia) or in the OGD chamber (for cells undergoing OGD). After 45 min loading, the existing DMEM was replaced with phenol-free DMEM with glucose (normoxic control) or without glucose (OGD). Rhodamine fluorescence was measured with a FLUOstar OPTIMA microplate reader (λₑₓ = 505 nm). After the first reading CCCP was added to depolarize
mitochondria completely. Data are expressed as a percentage of the intensity of the untreated control culture as follows:

\[
\% \Delta \Psi_{mSAMPLE} = \frac{(\text{Rhodamine fluorescence}_{SAMPLE} - \text{Rhodamine fluorescence}_{CCCP}) \times 100}{(\text{Rhodamine fluorescence}_{CONTROL} - \text{Rhodamine fluorescence}_{CCCP})}.
\]

**Electron paramagnetic resonance (EPR):**

For these experiments, neurons were cultured in 30 mm dishes and pre-treated with DZ, DZ+R, or regular medium. For measurement of ROS, I used the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen Science Transfer & Diagnostics, Elzach, Germany), as described previously (Mrakic-Sposta et al., 2012; Katakam et al., 2014). A 10 mM CMH solution was prepared in Krebs-Hepes buffer (KHB) containing 25 μM deferoxamine methane-sulfonate salt (DF) chelating agent and 5 μM sodium diethyldithiocarbamate trihydrate (DETC) at pH 7.4. Dishes were washed with KHB containing DF and DETC and allowed to stabilize in the temperature and gas controller Bio III unit for 20 min and then CMH was added (200 μmol/L). After 15 min, 20 μL of the supernatant was transferred into the EPR capillary tube (Noxygen Science Transfer & Diagnostics), and placed in an E-scan spectrometer (Bruker, Billerica, MA) for data acquisition. The reaction of the probe with the neuronal ROS generated a spectrum which was sequentially recorded for 5 min to calculate the ROS production rate. The EPR signal was proportional to the unpaired electron numbers and was transformed into micromoles per min (μmol min⁻¹). Neuronal lysates were collected from each
dish for protein analysis and normalization as described in the “Western blots” section.

**Statistical Analysis:**

All data are expressed as mean ± standard error of the mean (mean ± SEM) and analyzed using a one-way ANOVA (for experiments using more than 2 groups) and the Tukey post hoc test or a t-test (for experiments using 2 groups). A p < 0.05 was considered statistically significant. Information concerning sample sizes is presented in the figure legends.

**Results**

**Viability:**

At normoxia, none of the pharmacological treatments changed viability of neurons significantly, except DZ+T (Figure 7). When viability of neurons was measured 24 h after OGD, OGD decreased viability of neurons significantly compared with untreated neurons at normoxic conditions (Figure 8). Diazoxide preconditioning increased viability of neurons significantly compared with untreated OGD neurons. Adding R, W, R+W, or T along with DZ during preconditioning blocked the protective effects of DZ. Rapamycin and W alone did not affect viability at normoxia or after OGD compared with the untreated neurons at normoxia or after OGD (data not shown).
Figure 7: Viability of neurons that were untreated or treated with diazoxide (DZ) or a combination of DZ and rapamycin (R), wortmannin (W), R + W, or Torin 1 (T) at normoxia. DZ + T treatment group significantly affected viability at normoxia. n = 6 repetitions of the experiment from 6 independent cultures, 96 wells per treatment. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons. Data are expressed as mean ± SEM.
Figure 8: Viability of neurons that were untreated or treated with diazoxide (DZ) or a combination of DZ and rapamycin (R), wortmannin (W), R+W, or Torin -1 (T) after oxygen glucose deprivation (OGD). OGD significantly decreased viability compared with untreated neurons at normoxia. DZ increased viability significantly while adding R, W, R+W, or T during DZ preconditioning blocked preconditioning. n = 6 repetitions of the experiment from 6 independent cultures, 96 wells per treatment. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons; #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
**Quantification of proteins:**

At normoxia, only DZ+R pre-treatment significantly reduced the ratio of phosphorylated/total Akt compared with untreated neurons at normoxia. OGD significantly reduced the ratio of phosphorylated/total Akt compared with untreated neurons at normoxia. Diazoxide preconditioning rescued this effect by significantly increasing the ratio of phosphorylated/total Akt compared with untreated OGD neurons. Adding R, W, or R+W along with DZ during the preconditioning treatment prevented DZ from increasing the ratio of phosphorylated/total Akt (Figure 9).

At normoxia, no significant changes in the ratio of phosphorylated/total mTOR were observed. OGD significantly decreased the ratio of phosphorylated/total mTOR compared with untreated neurons maintained at normoxia. DZ preconditioning increased the ratio of phosphorylated/total mTOR significantly compared with untreated neurons after OGD. Adding R, W, or R+W along with DZ during the preconditioning treatment prevented the effect of DZ on mTOR (Figure 10).

At normoxia, no significant changes in the ratio of phosphorylated/total S6K were observed in any of the treatment groups. After OGD, untreated neurons showed a significant decrease in the ratio of phosphorylated/total S6K compared with untreated neurons maintained at normoxia. Diazoxide preconditioned neurons showed a significant increase in the ratio of phosphorylated/total S6K after OGD compared with untreated neurons after
OGD. Adding R, W, or R+W during DZ preconditioning prevented the increase in ratio of phosphorylated/total S6K seen with DZ alone (Figure 11).
Figure 9: Ratio of phosphorylated/total Akt in neurons. Oxygen glucose deprivation (OGD) dephosphorylated Akt, diazoxide (DZ) rescued the dephosphorylation. DZ + rapamycin (R), DZ + wortmannin (W), DZ+R+W all prevented the DZ-mediated rescue of phosphorylation. n = 4 repetitions of the experiments from 4 independent cultures, 8 dishes per group. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons; #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 10: Ratio of phosphorylated/total mTOR in neurons. Oxygen glucose deprivation (OGD) dephosphorylated mTOR, diazoxide (DZ) rescued the dephosphorylation. DZ + rapamycin (R), DZ + wortmannin (W), DZ+R+W all prevented the DZ-mediated rescue of phosphorylation. n = 4 repetitions of the experiments from 4 independent cultures, 8 dishes per group. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons; #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 11: Ratio of phosphorylated/total S6K in neurons. Oxygen glucose deprivation (OGD) dephosphorylated Akt, diazoxide (DZ) rescued the dephosphorylation. DZ + rapamycin (R), DZ + wortmannin (W), DZ+R+W all prevented the DZ-mediated rescue of phosphorylation. n = 4 repetitions of the experiments from 4 independent cultures, 8 dishes per group. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons; #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
**S6K-targeted siRNA:**

siRNA mediated knockdown of S6K did not affect neuronal viability at normoxic conditions in any group (Figure 12). After OGD, the S6K-siRNA treated neurons that remained untreated or were preconditioned with DZ showed a significantly decreased viability compared with DZ OGD neurons (Figure 13).

Western blotting for the ratio of phosphorylated/total S6K revealed that the siRNA treatment significantly reduced this ratio for untreated and DZ preconditioned neurons compared with DZ OGD neurons (Figure 14).
Figure 12: Viability of neurons at normoxia when treated with S6K-targeted siRNA. The siRNA treatment did not significantly alter viability at normoxia in untreated or diazoxide (DZ) - preconditioned neurons. n = 3 repetitions of the experiment from 3 independent cultures, 6 dishes per group. A one-way ANOVA and a Tukey post-hoc test were used. Data are expressed as mean ± SEM.
Figure 13: Viability of neurons after oxygen glucose deprivation (OGD) when treated with S6K-targeted siRNA. The siRNA treatment prevented diazoxide (DZ) from preconditioning. Untreated neurons with S6K siRNA showed about 50% death with OGD. n = 3 repetitions of the experiment from 3 independent cultures, 144 wells per treatment. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with DZ OGD neurons. Data are expressed as mean ± SEM.
Figure 14: Effect of S6K-targeted siRNA on ratio of phosphorylated/total S6K. Diazoxide (DZ) preconditioning increased ratio of phosphorylated/total S6K. S6K-targeted siRNA prevented DZ from increasing the phosphorylation of S6K. n = 3 repetitions of the experiment from 3 independent cultures, 6 dishes per group. A one-way ANOVA and a Tukey post-hoc test were used. #p < 0.05 compared with DZ OGD neurons. Data are expressed as mean ± SEM.
**ROS production:**

Untreated neurons showed a tendency to increase ROS production after OGD. Diazoxide preconditioning decreased ROS production significantly at normoxia, compared with untreated neurons. After OGD, DZ preconditioned neurons maintained the decreased ROS production. Interestingly, W, R, or R+W added during DZ preconditioning did not alter the DZ effect on ROS production (Figure 15). An acute 15 min application of DZ significantly increased ROS production in neurons (Figure 16) compared with untreated neurons.
Figure 15: Reactive oxygen species (ROS) production in neurons after preconditioning with diazoxide (DZ). Untreated neurons showed a tendency to increased ROS production after oxygen glucose deprivation (OGD). DZ preconditioning decreased ROS production at normoxia and maintained the decrease after OGD. The inhibitors wortmannin (W), rapamycin (R), or R+W are unable to prevent DZ from preconditioning. n = 6 independent cultures, 96 wells per treatment. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons; #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 16: Reactive oxygen species (ROS) production with a 15 min acute diazoxide (DZ) application. DZ application stimulated ROS production as measured using electron spin resonance. n = 6 repetitions of the experiment from 3 independent cultures, 6 dishes for each group. A t-test was used. *p < 0.05 compared with untreated control neurons. Data are expressed as mean ± SEM.
Mitochondrial Membrane potential:

OGD depolarized mitochondrial membrane potential in untreated neurons.

With a 3 day DZ preconditioning treatment, neurons maintained at normoxia showed a significantly decreased mitochondrial membrane potential compared with untreated neurons at normoxia. After OGD, DZ preconditioned neurons showed a significantly decreased mitochondrial membrane potential compared with untreated neurons after OGD. Addition of R, W, or R+W during DZ preconditioning did not significantly alter DZ’s effects on mitochondrial membrane potential at normoxia or after OGD (Figure 17). Rapamycin and W alone did not affect mitochondrial membrane potential. Acute application of DZ decreased mitochondrial membrane potential (Figure 18) significantly compared with untreated neurons.
Figure 17: Mitochondrial membrane potential in diazoxide (DZ) - preconditioned neurons. Untreated neurons showed a decreased membrane potential after exposure to oxygen glucose deprivation (OGD). DZ preconditioning decreased mitochondrial membrane potential at normoxic conditions and further after OGD. Adding rapamycin (R), wortmannin (W) or R+W during DZ preconditioning could not prevent the decrease in membrane potential seen with DZ alone. n = 6 repetitions of both experiments from 3 independent cultures, 288 wells per treatment for mitochondrial membrane potential measurement. A one-way ANOVA and a Tukey post-hoc test were used. *p < 0.05 compared with untreated control neurons; #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 18: Mitochondrial membrane potential changes with an acute, 15 min diazoxide (DZ) application. DZ decreased mitochondrial membrane potential and depolarized mitochondria in neurons after a 15 min acute treatment as measured using Rhodamine 123. n = 6 repetitions of both experiments from 3 independent cultures, 288 wells per treatment. A t-test was used. *p < 0.05 compared with untreated control neurons. Data are expressed as mean ± SEM.
Experiment 2: The role of neuronal nitric oxide synthase (nNOS) in DZ-induced preconditioning of primary rat cortical neurons.

In Experiment 2, I tested the hypothesis that the NOS pathway is also an important mediator of the DZ-induced preconditioning in neurons. I pharmacologically inhibited NOS using L-NAME. I also supplied NO exogenously using sodium SNP and DEANO. As in Experiment 1, I assessed cellular viability and protein expression of phosphorylated nNOS/total nNOS. Additionally, I examined whether the treatment groups showed any changes in phosphorylated S6K/total S6K protein expression to check for an interaction between the two pathways (Figure 19).
Figure 19: Treatment protocol for Experiment 2. Primary neurons were cultured from E-18 rats. After 6 days, neurons were treated for 3 days consecutively with diazoxide, or diazoxide + L-NAME, diazoxide + L-NAME + SNP, or diazoxide + L-NAME + DEANONOate (DEANO). After this, cells were placed in an anaerobic chamber to mimic an ischemic stroke. During reoxygenation, cells were collected for viability assays and western blots.
**Methods:**

**Sterile Technique:**
Similar to Experiment 1, sterile technique was maintained.

**Animals:**
Pregnant, female rats as described in Experiment 1 were used for this experiment.

**Rat Primary Cortical Neuronal Cell Culture:**
Neurons were cultured the same as in Experiment 1

**Materials:**
Diazoxide was used as described in Experiment 1. L-NAME (100μM, Sigma) dissolved in sterile water was used to inhibit NOS. SNP (1 μM, Sigma) was dissolved in water and DEANONOate (5 μM, Sigma) was dissolved in DMSO.

**Experimental Design:**
For Experiment 2, primary cortical neurons were cultured for 7 days in vitro (DIV) in 3mm dishes and 96-well plates. On the 7th day, cells were treated with (1) DZ, (2) DZ+L-NAME, (3) DZ+L-NAME+SNP, or (5) DZ+L-NAME+DEANONOate for 3 consecutive days. On DIV 10, neurons were either subjected to OGD or maintained at normoxia, as described below. Mitochondrial membrane potential and ROS measurements were conducted immediately after OGD. Lysates were collected after 2 h of reoxygenation for Western blotting and viability assays and mitochondrial respiration assays were performed 24 h after reoxygenation.
**Oxygen Glucose Deprivation (OGD):**

OGD was used to model an *in vitro* stroke as described in Experiment 1.

**Quantification of Cellular Viability Assay:**

Viability assays were conducted as described in Experiment 1.

**Western Blots:**

Proteins were collected and Western blots were performed as described in Experiment 1. The phosphorylated nNOS $^{\text{Ser1417}}$ (serine 1417, p-nNOS, 160KDa, Abcam), and total nNOS (160 KDa, BD transduction) antibodies were used.

**Statistical Analysis:**

The same statistical analyses were followed in this experiment as described in Experiment 1.

**Results**

**Viability:**

At normoxia, none of the treatments significantly affected neuronal survival compared with untreated neurons (Figure 20).

After OGD, untreated neurons showed a significant decrease in viability. DZ preconditioning improved neuronal survival significantly after OGD, compared with untreated neurons. Adding L-NAME along with DZ during preconditioning abolished DZ’s preconditioning effects. Exogenously supplying NO using donors such as SNP and DEANONOate along with the DZ and L-NAME rescued DZ’s protective effects. This finding suggests that the NOS signaling pathway plays a critical role in DZ-afforded preconditioning and neuroprotection (Figure 21).
**Quantification of proteins:**

DZ+L-NAME treated neurons showed a significantly decreased ratio of phosphorylated/total nNOS and S6K expression with OGD (figure 22, 25). The ratio of phosphorylated/total Akt and mTOR remained unchanged in DZ+L-NAME treated neurons after OGD (figure 23, 24). At normoxia there were no significant changes in the proteins examined.
Figure 20: Viability of neurons at normoxia after modulating the neuronal nitric oxide synthase (nNOS) pathway. Diazoxide (DZ), DZ+L-NAME, DZ+LNAME+SNP, and DZ+L-NAME+DEANO do not affect neuronal viability significantly compared with untreated neurons. n = 6 repetitions of both experiments from 3 independent cultures, 16-32 wells per treatment. Data are expressed as mean ± SEM.
Figure 21: Viability of neurons after oxygen glucose deprivation (OGD) with similar pharmacological treatments as Figure 18. OGD significantly reduced neuronal viability in the untreated group compared with untreated neurons at normoxia. A similar significant decrease in viability was observed when L-NAME was added along with diazoxide (DZ) during the preconditioning treatment. DZ preconditioning increased neuronal viability compared with untreated neurons. Supplying NO via NO donors SNP and DEANONOate along with DZ and L-NAME also increased viability significantly after OGD compared with untreated neurons. n = 6 repetitions of both experiments from 3 independent cultures, 16-32 wells per treatment. *p < 0.05 compared with untreated control neurons. #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 22: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total nNOS. Diazoxide (DZ) preconditioning led to a significant increase in the ratio of phosphorylated/total nNOS in neurons compared with untreated OGD neurons. Using L-NAME to block all NOS abolished the effect of DZ. n = 3 repetitions from 3 independent cultures, *p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 23: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total Akt. Diazoxide (DZ) preconditioning led to a significant increase in the ratio of phosphorylated/total Akt in neurons compared with untreated oxygen glucose deprivation (OGD) neurons. Using L-NAME to block all NOS abolished the effect of DZ. n = 6 repetitions from 3 independent cultures, #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 24: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total mTOR. Diazoxide (DZ) preconditioning led to a significant increase in the ratio of phosphorylated/total Akt in neurons compared with untreated oxygen glucose deprivation (OGD) neurons. Using L-NAME to block all NOS abolished the effect of DZ. n = 6 repetitions from 3 independent cultures, #p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 25: Effect of pharmacologically inhibiting neuronal nitric oxide synthase (nNOS) using L-NAME in neurons on the ratio of phosphorylated/total S6K. Diazoxide (DZ) preconditioning led to a significant increase in the ratio of phosphorylated/total S6K in neurons compared with untreated oxygen glucose deprivation (OGD) neurons. Using L-NAME to block all NOS abolished the effect of DZ. n = 3 repetitions of both experiments from 3 independent cultures, *p < 0.05 compared with untreated OGD neurons. Data are expressed as mean ± SEM.
Experiment 3: Characterization of mitochondrial respiration in DZ-preconditioned neurons.

Finally, in Experiment 3 I tested the hypothesis that mitochondrial respiration is altered with DZ-induced preconditioning. I used the Seahorse Xfe 24 analyzer to modify the electron transport chain in mitochondria of intact neurons by serially injecting oligomycin, FCCP, and antimycin/rotenone. I assessed respiration 24 and 48 h after OGD as well as with acute DZ injections (Figure 26).
Figure 26: Treatment protocol for Experiment 3. Primary neurons were cultured from E-18 rats. After 6 days, neurons were treated for 3 days consecutively with diazoxide (DZ). Following this, cells were placed in an anaerobic chamber to mimic an ischemic stroke. At 24 and 48 h after reoxygenation Seahorse Mito-Stress Tests were conducted to estimate oxygen consumption rate (OCR). For the acute treatment, DZ was directly injected onto neurons in the Seahorse Analyzer.
Methods:

Sterile Technique:
Sterile technique was maintained as described in Experiment 1.

Animals:
Pregnant, female rats as described in Experiment 1 were used for this experiment.

Rat Primary Cortical Neuronal Cell Culture:
Neurons were cultured similarly to neurons in Experiment 1.

Materials:
Diazoxide was used as described in Experiment 1. For mitochondrial respiration measurements, 2 µM oligomycin (ATP synthase inhibitor, Seahorse Biosciences, Billerica, MA, USA), 3.5 µM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, an ionophore, Seahorse Biosciences), 1.5 µM antimycin (complex III inhibitor, Seahorse Biosciences), and 1.5 µM rotenone (a complex I inhibitor, Seahorse Biosciences) were dissolved in DMSO.

Poly-D-lysine coating:
Poly-D-lysine (Sigma) was dissolved in 50mL sterile tissue culture grade water. This was transferred to a pre-sterilized bottle, and another 50mL of the sterile tissue culture grade water was added. The bottle was swirled to ensure mixing and the final concentration was 50 µg/mL. 5ml aliquots was made for storage in -20° C. Laminin (Sigma) was thawed slowly on ice and added to the poly-D-lysine (10 µL laminin/1ml of poly-D-lysine). 50 µL of this mixture was added to each well of the 24-well Seahorse plate and incubated overnight at 37°C.
C. The following day the solution was aspirated and rinsed 3 times with 500 µL sterile tissue culture grade water. The plates were left wet until immediately before cell plating.

**Experimental Design:**

Primary cortical neurons were cultured for 7 days *in vitro* (DIV). On the 7th day, cells were treated with regular feeding medium or DZ for 3 consecutive days. On DIV 10, neurons were subjected to OGD or maintained at normoxia, as described below.

**Oxygen Glucose Deprivation:**

OGD was used to model an *in vitro* stroke as described in Experiment 1.

**Mitochondrial respiration of neurons:**

Oxygen consumption rate (OCR) was measured using a Seahorse XF® 24 analyzer as described previously (Yao et al., 2013; Yao et al., 2012; Yao et al., 2009; Yao et al., 2011a; Yao et al., 2011b). Specially designed, poly-D-lysine coated 24-well plates were used to measure mitochondrial respiration in the cultured neurons. Fluorescent sensors measured the oxygen and proton fluxes present in the microchamber above the cells, which allowed calculation of the OCR. Neurons were washed twice with Assay Medium (Seahorse Biosciences) supplemented with 5mM glucose (Sigma Aldrich) and 2mM pyruvate (Sigma Aldrich). Mitochondrial electron transport chain function was altered using serial injections of the drugs oligomycin, FCCP, and antimycin plus rotenone (Figure 27). Each assay was normalized to and expressed as a percentage of the baseline value.
**Figure 27:** Injections and oxygen consumption rate (OCR) measurement in neurons using Seahorse Xfe24 Analyzer. Neurons plated on specially coated 24-well plates were serially injected with oligomycin, FCCP, and antimycin/rotenone after basal OCR measurement. Each step had 3 points of measurement and each point had a 3 min mixing period, followed by a 5 min waiting period and finally a 3 min measuring period.
**Statistical Analysis:**

The same statistical analyses were followed in this experiment as described in Experiment 1.
Results

There were no significant differences in OCR between DZ treated and control neurons under normoxic conditions without OGD (Figure 28, 30-35). The OCR was consistently higher in DZ compared with untreated neurons 24 h after OGD (Fig 29, 30-35).

After normalization of OCR values to baseline values for DZ treated and control neurons, DZ-treated neurons subjected to OGD showed significantly elevated OCR 24 and 48 h post-OGD following oligomycin, FCCP, and antimycin/rotenone injection compared with untreated neurons at 24 and 48 h post-OGD.

Acute application of DZ for 15 min did not alter OCR significantly in neurons (Figure 36). Interestingly, the same acute treatment with DZ for 15 min significantly increased extracellular acidification rate (ECAR) of neurons (Figure 37).
Figure 28: Representative tracing from Seahorse Mito-stress test on preconditioned neurons. At normoxia, there was a tendency for diazoxide (DZ) - preconditioned neurons to have lower oxygen consumption rate compared with the untreated neurons.
Figure 29: Representative tracing from Seahorse Mito-stress test on preconditioned neurons 24 h after OGD. After oxygen glucose deprivation (OGD), there was a tendency for diazoxide (DZ) - preconditioned neurons to have higher oxygen consumption rate compared with the untreated neurons.
Figure 30: Effect of oligomycin injection on oxygen consumption rate (OCR) in neurons 24 h after oxygen glucose deprivation (OGD). At normoxia, no significant differences were observed between untreated and diazoxide (DZ) - preconditioned neurons. 24 h after OGD DZ-preconditioned neurons displayed a significantly higher OCR compared with untreated OGD neurons upon oligomycin injection. n = 8 animals, 80 wells per treatment. #p < 0.05 vs. untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 31: Effect of FCCP injection on oxygen consumption rate (OCR) in neurons 24 h after oxygen glucose deprivation (OGD). At normoxia, no significant differences were observed between untreated and diazoxide (DZ) - preconditioned neurons. 24 h after OGD DZ-preconditioned neurons displayed a significantly higher OCR compared with untreated OGD neurons after FCCP injection. n = 8 animals, 80 wells per treatment. #p < 0.05 vs. untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 32: Effect of antimycin/rotenone injection on oxygen consumption rate (OCR) in neurons 24 h after oxygen glucose deprivation (OGD). At normoxia, no significant differences were observed between untreated and diazoxide (DZ) - preconditioned neurons. 24 h after OGD, DZ-preconditioned neurons displayed a significantly higher OCR compared with untreated OGD neurons after antimycin/rotenone injection. n = 8 animals, 80 wells per treatment. #p < 0.05 vs. untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 33: Effect of oligomycin injection on oxygen consumption rate (OCR) in neurons 48 h after oxygen glucose deprivation (OGD). At normoxia, no significant differences were observed between untreated and diazoxide (DZ) - preconditioned neurons. 24 h after OGD DZ-preconditioned neurons displayed a significantly higher OCR compared with untreated OGD neurons after FCCP injection. n = 8 animals, 80 wells per treatment. #p < 0.05 vs. untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 34: Effect of FCCP injection on oxygen consumption rate (OCR) in neurons 48 h after oxygen glucose deprivation (OGD). At normoxia, no significant differences were observed between untreated and diazoxide (DZ) - preconditioned neurons. 24 h after OGD DZ-preconditioned neurons displayed a significantly higher OCR compared with untreated OGD neurons after FCCP injection. n = 8 animals, 80 wells per treatment. #p < 0.05 vs. untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 35: Effect of antimycin/rotenone injection on oxygen consumption rate (OCR) in neurons 48 h after oxygen glucose deprivation (OGD). At normoxia, no significant differences were observed between untreated and diazoxide (DZ) - preconditioned neurons. 24 h after OGD DZ-preconditioned neurons displayed a significantly higher OCR compared with untreated OGD neurons after FCCP injection. n = 8 animals, 80 wells per treatment. #p < 0.05 vs. untreated OGD neurons. Data are expressed as mean ± SEM.
Figure 36: Acute oxygen consumption rate (OCR) in neurons. A 15 min application of media, vehicle (0.1 M NaOH) or diazoxide (DZ) did not significantly change mitochondrial respiration using OCR. Data are expressed as mean ± SEM.
Figure 37: Acute extracellular acidification rate (ECAR) in neurons. A 15 min application of diazoxide (DZ) significantly increased the ECAR in neurons compared with vehicle application. *p < 0.05 vs. vehicle treated neurons. Data are expressed as mean ± SEM.
Conclusions and Discussion

Conclusions

There are three new, significant findings from our studies. First, I have presented evidence that the Akt-mTOR-S6K cascade plays an important role in mediating DZ-induced preconditioning. Although previous studies have suggested that chronic mitochondrial depolarization and decreased ROS production are important DZ-induced preconditioning mechanisms during OGD, the current study elucidates the involvement of other supporting pathways. Second, I have shown that the NOS pathway plays an important role in preconditioning by DZ. Finally, I have found that increased cell survival after OGD is associated with modified mitochondrial and non-mitochondrial respiration in DZ-preconditioned neurons. Thus, the integration of mTOR and NOS pathways, and altered mitochondrial respiration after DZ-induced preconditioning results in augmented S6K activity and neuronal protection.

Experiment 1

Diazoxide increased neuronal viability 24 h after OGD. By using two structurally different inhibitors, R and T, I have demonstrated that the mTOR complex is essential for DZ to exert its protective effects against the ischemic insult to neuronal cells. The addition of the pharmacological inhibitor of PI3K, W,
also prevented DZ-induced preconditioning. In addition, these studies have shown the robust nature of DZ preconditioning. The 3-day treatment was protective after DZ was washed out of the neurons and the protection was seen 48 h after the last treatment with DZ.

Activation of the mTOR pathway has also been implicated in several disorders of the nervous system. Li and colleagues (2010b) showed that mTOR activation is involved in the beneficial effects of ketamine in the acute treatment of depression. In addition, Kumar et al (2013) reported that mTOR activation promoted remyelination of nerves in a multiple sclerosis mouse model. Moreover, the mTOR complex and its main downstream kinase, S6K, are thought to be key players in the outcome of an ischemic insult to neurons and astrocytes as shown in both in vitro and in vivo models (Xie et al., 2013; Mao et al., 2013; Wang et al., 2012; Shi et al., 2011; Chen et al., 2012a; Pastor et al., 2009). Our results are in agreement with several other studies demonstrating that neuroprotective agents such as PTEN inhibitor BPV, melatonin, dehydroepiandrosterone, and atorvastatin can activate the mTOR pathway (Mao et al., 2013; Koh, 2008; Li et al., 2010a; Jin et al., 2012). Also, similar to our current findings, Kis, Yellon and Baxter (2003a) demonstrated that DZ preconditioning in cardiac tissue activates the mTOR pathway. In cardiac tissue, the mTOR pathway has been shown to be crucial for coronary artery growth which is also known to affect outcomes after ischemia (Pung et al., 2013). A few studies have shown that down-regulation of the mTOR pathway during an ischemic event is beneficial by inducing autophagy.
(Urbanek et al., 2014; Fletcher et al., 2013). Conflicting results from other studies may be the result of different methodologies used.

**Experiment 2**

Our study has uncovered a novel role of NOS in DZ-induced preconditioning. Inhibiting NOS with L-NAME during DZ-induced preconditioning disrupts protective signaling suggesting that NO is essential for preconditioning with DZ. We previously showed that DZ acutely induces NOS phosphorylation and NO production in endothelial cells from cerebral arteries (Katakam et al., 2013). Nitric oxide is known to have cytoprotective effects, mainly by post-translational modification of various proteins via S-nitrosylation, upregulation of cGMP signaling, and peroxynitrite formation (Iadecola et al., 2011). Recent studies in the heart have shown that S-nitrosylation, not cGMP signaling, is important in the protective effects imparted by IPC (Sun et al., 2015; Sun et al., 2013). Moreover, NO signaling has been implicated in early LPS-induced preconditioning and delayed hypoxic preconditioning (Orio et al., 2007). Similar results have been published using desflurane post-conditioning of isolated human atria (Lemoine et al., 2010), ischemic preconditioning of the retina (Zhu et al., 2006), and isoflurane-induced cardioprotection (Krolikowski et al., 2006). Our data also indicate that NO production by nNOS during preconditioning protects against subsequent nNOS mediated neuronal cell death during OGD. Giedt and colleagues also shed light on how nitrosative and oxidative stress can enhance mitochondrial fission in ischemia-reperfusion injury (Giedt et al., 2012).
Relatively few studies have addressed the relationship between the mTOR and NOS signaling pathways. Espana and colleagues (2013) demonstrated that nNOS is upstream of mTOR in a murine model of autoimmune blistering skin disease. Fruhwurth et al. (2014) also showed that R inhibits NO production and leads to endothelial dysfunction in human umbilical vein endothelial cells. In addition, Berven LA, Frew IJ, and Crouch MF (1999) showed that NO induces phosphorylation of S6K. Finally, Barkoudah et al (2004) and Castrop et al (2004) have shown that NO exerts a permissive effect on signaling pathways in different cell types. In support of these studies, I have shown that the ratio of phosphorylated/total S6K is downregulated in DZ+L-NAME treated neurons, indicating probable crosstalk between the Akt-mTOR-S6K and NO pathways. Interestingly, the ratios of phosphorylated/total Akt and mTOR are unaffected by L-NAME addition during preconditioning.

**Experiment 3**

Although it is well-established that DZ protects mitochondrial integrity and calcium handling after anoxia (Busija and Katakam, 2014), this is the first report showing elevated OCR in response to DZ treatment 24 h after an anoxic event, indicating a more robust electron transport chain function in preconditioned neurons and/or increased non-mitochondrial oxygen consumption. During normoxia, vehicle and DZ treated neurons behaved similarly and showed the normal profile observed in most cell types after administration of oligomycin, FCCP, and antimycin/rotenone. Thus, in both groups during normoxia, oligomycin reduced OCR by more than 60%, FCCP increased OCR by more
than 50%, and antimycin/rotenone decreased OCR by approximately 80%. Since OCR values were almost identical in the vehicle and DZ treated neurons during normoxia, despite a dramatically reduced mitochondrial membrane potential and ROS production in the DZ group, membrane potential and ROS availability exerted minimal effects on OCR in healthy neurons. However, OGD changed the OCR profiles for both treatment groups. First, vehicle treated neurons showed a 50% reduction in OCR following oligomycin whereas DZ treated neurons showed a smaller drop in OCR (about 40%). Therefore, at baseline, ATP synthesis may be driven by oxidative phosphorylation, but after an ischemic insult there is an uncoupling effect which is more pronounced in the DZ preconditioned neurons. A similar uncoupling effect has been reported previously by mitoK\textsubscript{ATP} activation (Costa et al., 2006; Pamenter et al., 2008). Second, adding FCCP, which allowed us to determine the maximal respiratory capacity, showed reductions in OCR for vehicle and DZ treated neurons after OGD compared with normoxia. The reduction in OCR, however, was less in the DZ treated neurons indicating that OGD exhausts the maximal capacity of neurons and DZ treatment allows for a partial preservation of the maximal capacity. Third, antimycin/rotenone administration reduced OCR less after OGD than in normoxic neurons, indicating increased involvement of non-mitochondrial mechanisms. Therefore, after OGD, neurons appear to consume more oxygen via non-mitochondrial enzymes, especially in DZ treated neurons.

Next, the same assays conducted on neurons 48 h after OGD showed similar results. These data reflect the powerful and long-lasting effect DZ
preconditioning has on neuronal mitochondria; the neurons in the 24 and 48 h group were last treated with DZ 48 and 72 h before the Seahorse assay.

In neurons undergoing an acute or 15 min application of DZ, no changes in mitochondrial respiration were observed. This is not surprising, since the dual effects of DZ on increasing mitochondrial respiration by depolarizing them and also inhibiting complex II, may ultimately balance each other out. Such results have been reported prior to this, suggesting that at the doses I have used to precondition neurons, both effects of DZ are prominent. An interesting finding that demands further investigation is the increased extracellular acidification rate (ECAR) with acute DZ application. This may suggest that acutely, DZ application leads to a switch towards glycolysis.

I considered the involvement of oxygen consuming reactions such as those involving nitric oxide synthase (NOS), cyclooxygenase (COX), and heme-oxygenase (HO). Specifically, our lab has shown that the depolarization of mitoK<sub>ATP</sub> channels using DZ leads to activation of the NOS pathway in cultured endothelial cells and cerebral blood vessels (Rutkai et al., 2014; Katakam et al., 2013). Additionally, Alcindor et al. (2004) concluded that COX-2 mediates the protection imparted by DZ in pharmacological preconditioning in a canine model of myocardial infarction. Zeng and colleagues (2012) also showed that DZ pre-treatment enhanced HO-1 expression which led to increased resistance to liver ischemia-reperfusion injury in rodents. Although certain cytosolic enzymes such as NADPH oxidases and cytochrome P450 have not been studied extensively with regard to DZ preconditioning there is some literature suggesting cross-talk
between mitochondria and NADPH oxidases (Nazarewicz et al., 2013; Dikalov, 2011; Bedard and Krause, 2007) and between vasorelaxant effects of DZ and cytochrome P450 (Oyekan et al., 1994). Santos and colleagues (2002) have shown that in the ischemic heart, DZ pre-treatment allows for a more efficient energy transfer between mitochondrial and non-mitochondrial enzymes upon reperfusion. A reduction in oxygen consumption has also been shown in Alzheimer’s disease (Yao et al., 2011a), ischemia-reperfusion injury (Porter et al., 2014), and hypoxic insult of cardiomyocytes (Neary et al., 2014). Finally, Kelly et al. (2014) reported that the inhibition of the PI3K-mTOR pathway decreases OCR in several cancer cell lines, supporting our premise that this axis is important for DZ-induced preconditioning. Further studies are needed to confirm that mitochondrial respiration is enhanced and the mTOR pathway is activated in vivo by pre- and post-conditioning with DZ.
Limitations

Although this research and its aims were carefully prepared, there are some unavoidable limitations in all methods. I have used only primary cortical neurons from rats which represents a very specific population of neurons. Although they express all the necessary receptors, they are derived from young embryos and are subjected to a stroke on DIV 7. We may see different results in more mature cultures. It is known that in humans, strokes affect both gray and white matter and multiple cell types and blood vessels. Also, I did not culture male or female neurons separately, thus I could not explore whether there are any sex-based differences in neuronal preconditioning using DZ. With the existing study design, we looked only at changes after a 3-day treatment and we may have observed other differences at other time points.

As already mentioned, the pathways studied in this dissertation are complex and I used multiple approaches to target different parts of each pathway. For the mTOR pathway, I used two distinct inhibitors for mTOR: the canonical rapamycin and the newer Torin-1 that gives better inhibition of complex II. I also used wortmannin to further verify the pathway involvement. It is important to note that all drugs have non-specific effects. I further confirmed the involvement of the mTOR pathway by using siRNA targeting S6K. Transfecting
neurons using siRNA is difficult as they are post-mitotic and already plated at 100% confluency for our studies. Ideally, siRNA should be used on cells that are actively dividing and plated at 60-70% confluency for optimal transfection efficiency. Despite this limitation, I saw a significant effect on the viability and ratio of phosphorylated/total S6K. Similarly, with the nNOS studies I used L-NAME which is a general NOS inhibitor that is most selective for eNOS and nNOS. To confirm NO involvement, I used exogenous donors such as SNP and DEANO, both of which work with different mechanisms.

I used Western blotting to understand the various proteins that are involved in preconditioning neurons using DZ. It is recognized that Western blots are semi-quantitative and do not reflect the activity of these proteins. Additionally, antibodies are often selective to more than the protein of interest allowing background effects that make analysis difficult.

I used OGD as our model for ischemic stroke. This is a well-established model to mimic stroke in an in vitro situation but lacks the complexity seen in a whole-animal model of stroke such as MCAO. For example, our cultures are composed predominantly of neurons, which eliminates a lot of inter- and intra-cellular cross-talk. Also I could not see an ischemic core and penumbra development as is commonly seen in stroke.

After OGD I used a mitochondrial dehydrogenase based viability assay to measure neuronal death. The assay itself is reproducible and well-studied but certain nuances of cell death are lost. For example, I cannot conclude which
neurons died due to apoptosis vs. necrosis or why 50% of the neurons remained alive.

I used electron spin resonance to detect ROS production from intact cells. Although this method is highly sensitive I noticed that sometimes the spin probe and the drug of interest interacted with each other which may have confounded results. CMH, the probe I used, is most specific to superoxide.

I used a fluorescent dye, Rhodamine 123, to measure mitochondrial depolarization. At present this method does not allow patching onto intact mitochondria to directly measure mitochondrial membrane potential. However, the dyes of the Rhodamine family have been criticized for binding differently depending on the energy status of the mitochondria, which may confound results (Salvioli et al., 1997).

To measure mitochondrial respiration in intact neurons, I used the Seahorse analyzer. However, I only investigated net oxygen consumed by both mitochondrial and non-mitochondrial processes. I was unable to gather information about glycolysis or which non-mitochondrial processes were specifically affected by the preconditioning. The mito-stress assay itself is very time consuming. ATP production kits have shorter incubation times and may have yielded different results.
Implications and future directions

In conclusion, my findings show that changed mitochondrial respiration is an important component of neuronal survival after OGD and support the hypothesis that the mTOR and NOS pathways are activated after DZ-induced preconditioning (Figure 38). I also observed that DZ preconditioning effects are not limited to mitochondria; there is significant cross-talk between the mitochondria and cellular signaling pathways. The mTOR pathway and NOS signaling appear to be interconnected and there are multiple feedback and parallel pathways for both. For example, mTORC2 is known to activate Akt by phosphorylation. Thus, rapamycin alone incompletely inhibits mTORC1 in an acute setting and only partially affects translation of new proteins. In fact, rapamycin has been shown to upregulate survival of cancer cells due to inhibition of S6K dependent feedback loops that regulate PI3k and Akt (Zoncu et al., 2011). On the other hand, Torin-1 is a dual inhibitor that affects both complexes and thus yields slightly different results than rapamycin alone.

Similarly NO-NOS is known to have a certain degree of feedback regulation that affects the expression and activity of NOS enzymes (Kopincova et al., 2011). Thus, it is important to appreciate that cellular pathways such as
mTOR and NOS are not linear or mutually exclusive, but represent very complex interactions and convergence of signals.

The Seahorse experiments would benefit from extrapolation to the Glycolysis Stress Test to see whether other modes of respiration are also affected with acute and preconditioning DZ treatments.

Finally, these experiments also display the robust and long-term benefits provided by preconditioning with an agent such as DZ. These benefits suggest that investigating post-translational modifications after DZ preconditioning could lead to more targets for treatment of ischemic strokes.

The current therapeutic options, tPA and mechanical clot removal, are exceedingly limited in use (Kirkman et al., 2014). Our studies in neurons using a DZ-preconditioning model against OGD suggest that the mTOR and NOS pathways as well as mitochondria are potential targets for therapies for high-risk patients.
Figure 38: Proposed signaling schematic of diazoxide (DZ) preconditioning. Diazoxide preconditioning affects mitochondrial parameters, i.e., ROS production, membrane potential, and oxygen consumption. Using pharmacological and siRNA techniques, I found that DZ preconditioning also affects the PI3K-Akt-mTOR-S6K and NOS signaling and also alters non-mitochondrial oxygen consumption.
References


Chen H, Xiong T, Qu Y, Zhao F, Ferriero D, Mu D (2012b) mTOR activates hypoxia-inducible factor-1alpha and inhibits neuronal apoptosis in the developing rat brain during the early phase after hypoxia-ischemia. Neurosci Lett (Ireland) 507:118-123.


Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA (1996) Enlarged infarcts in endothelial nitric oxide synthase knockout mice are


Katakam PV, Domoki F, Snipes JA, Busija AR, Jarajapu YP, Busija DW (2009) Impaired mitochondria-dependent vasodilation in cerebral arteries of zucker
obese rats with insulin resistance. Am J Physiol Regul Integr Comp Physiol (United States) 296:R289-98.


Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM,


Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS (2010b) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. Science (United States) 329:959-964.


Salvioli S, Ardizzoni A, Franceschi C, Cossarizza A (1997) JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: Implications for studies on mitochondrial functionality during apoptosis. FEBS Lett (NETHERLANDS) 411:77-82.


Thompson JW, Dave KR, Young JI, Perez-Pinzon MA (2013) Ischemic preconditioning alters the epigenetic profile of the brain from ischemic intolerance to ischemic tolerance. Neurotherapeutics (United States) 10:789-797.


Yao J, Chen S, Mao Z, Cadenas E, Brinton RD (2011b) 2-deoxy-D-glucose treatment induces ketogenesis, sustains mitochondrial function, and reduces


Biography

Somhrita Dutta was born in Calcutta, India on February 7, 1988 to Swapan Kumar Dutta and Anulekha Dutta. She grew up with her older brother Shuvam Dutta and dog, Leo. She finished her schooling up until high school at Ashok Hall Girls Higher Secondary School in Calcutta, India. Following this, she attended Colorado College in Colorado Springs, CO, USA and received her Bachelor of Arts’ in Neuroscience in 2010. She has been actively pursuing a PhD in Neuroscience at the Department of Pharmacology at Tulane University, New Orleans, LA, USA under the mentorship of Dr. David W. Busija.